NOTE
Avian Pathology

Avian Influenza Virus and Paramyxovirus Isolation from Migratory Waterfowl and Shorebirds in San-in District of Western Japan from 2001 to 2008

Yoshikazu FUJIMOTO1, Hiroshi ITO1,2, Sakar SHIVAKOTI1, Jyunya NAKAMORI2, Ryota TSUNEKUNI3, Koichi OTSUKI2,3 and Toshihiro ITO1,2*

1)Laboratory of Veterinary Public Health and 2)Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, 101 Minami 4-chome Koyama-cho, Tottori 680–8553 and 3)Avian Influenza Research Center, Kyoto Sangyo University, Motoyama, Kamigamo, Kita-Ku, Kyoto 603–8555, Japan

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ABSTRACT. Surveillance of avian influenza virus and paramyxovirus in migratory waterfowl and shorebirds was conducted in the San-in district of western Japan from the winter of 2001 to 2008. From 4,335 fecal samples from wild birds, 41 avian influenza viruses of 12 different HA and NA combinations, including two H5N3 strains, and 13 avian paramyxoviruses were isolated. Phylogenetic analysis of HA genes revealed that H5N3 strains clustered in a different branch from the recent highly pathogenic H5N1 isolates in Japan; however, the introduction of new highly pathogenic avian influenza virus by migratory birds cannot be ignored. Therefore, it is necessary to continue surveillance of these potentially serious pathogens in waterfowl and shorebirds.

KEY WORDS: avian influenza virus, avian paramyxovirus, migratory waterfowl, shorebird.

Avian influenza virus (AIV) belongs to the family Orthomyxoviridae and has two types of surface glycoprotein, hemagglutinin (HA) and neuraminidase (NA), which are divided into H1 to H16 and N1 to N9 subtypes based on their antigenic specificity [4, 20]. If poultry, such as chickens and turkeys, are infected with AIVs, they exhibit various disease symptoms, ranging from mild respiratory signs caused by low pathogenic avian influenza viruses (LPAIVs) to high mortality induced by highly pathogenic avian influenza viruses (HPAIVs), which are restricted to H5 and H7 subtypes; therefore, AIVs are considered to be the most important diseases with regard to economic damage to the poultry industry.

In addition to AIV, avian paramyxovirus (APMV) is a virus that causes an important disease impacting international trade in poultry and poultry products. APMV belongs to the family Paramyxoviridae and has nine antigenic serotypes (APMV-1 to -9) based on their hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assay results [1]. Very little is known about the molecular and biological characteristics and pathogenicity of APMV serotypes 2–9, while APMV-1, known as Newcastle disease virus (NDV), has been studied extensively. NDVs are divided into three major pathotypes based on their pathogenicity in chickens: lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (high virulence) [1]; therefore, outbreaks of Newcastle disease, similarly to avian influenza, have resulted in severe economic losses in the poultry industry.

Wild birds, particularly waterfowl, are a reservoir of all known AIV subtypes other than H13 and H16 strains, which are found exclusively in shorebirds, such as gulls [4, 20], and APMV-1, -4, -6, -8 and -9 serotypes [1]. Wild birds, including waterfowl and shorebirds, are thus considered to be important carriers for the transmission of AIVs and APMVs. In Japan, waterfowl such as geese, swans and ducks are well-known wintering migratory birds. These birds fly to Japan from Alaska, the Russian Far East, eastern Siberia, eastern Mongolia, and northeastern China. Since December 1979, we have conducted continued surveillance of AIVs in migratory Anseriformes (e.g., ducks, geese and swans) and Charadriiformes (e.g., gulls) in western Japan [10–13, 15, 17]. No highly pathogenic AIVs have been isolated, but many subtypes of viruses from various species of wild birds have been detected. In a previous study, we experimentally demonstrated that non-pathogenic H5N3 AIV and APMV-1 (NDV) isolated from wild waterfowl became highly pathogenic after several cycles of infection in chickens [7, 14], thus suggesting that wild birds are able to transmit and spread viruses that are potential precursors for highly pathogenic derivatives in poultry birds. Therefore, continuous surveillance of AIVs and APMVs in wild birds, particularly waterfowl, is important for providing information on the prevalent subtypes of the viruses circulating in the field and emerging highly pathogenic viruses.

In this study, we surveyed AIVs and APMVs in wild waterfowl and shorebirds in winter during their migratory seasons from 2001 to 2008 in the San-in district of western Japan. Forty-one strains of AIV and 13 strains of APMV were isolated. Among the AIVs, two strains were identified as H5 subtypes and the phylogenetic tree of HA genes was constructed.

A total of 4,335 fresh fecal samples were collected from whistling swans (Cygnus columbianus jankowski), mallards (Anas platyrhynchos), common teals (Anas crecca), Eurasian wigeons (Anas penelope), northern pintails (Anas acuta), gadwalls (Anas strepera), white-fronted geese...
(Anser albinus), black-tailed gulls (Larus crassirostris) and duck spp. (Anas spp.) during winter (November-March) of fiscal years 2001 to 2008 (fiscal year 2001, for example, refers to the period from November 2000 through March 2002). Samples were collected from eight different sites: Lake Koyama, Pond Nikko, Lake Togo, Tenjin River, Hino River, Ito Coast, the Yonago waterbird sanctuary and rice fields of the suburbs of Yasugi City in the San-in district (Tottori and Shimane Prefectures) of western Japan. Fecal samples were collected individually into screw-capped tubes and stored at –80°C until assayed.

Virus isolation was performed as described previously [15] with slight modification. Fecal samples were suspended at a concentration of approximately 30% in phosphate-buffered saline (pH 7.2) containing penicillin at 10,000 units per ml and streptomycin at 10 mg per ml. The suspension was centrifuged at 1,000 × g for 10 min. Two hundred microliters of supernatant was inoculated into the allantoic cavities of two 9- to 11-day-old embryonated chicken eggs and were incubated at 37°C, their allantoic fluids were tested for hemagglutination activity.

All hemagglutinin agents were tested by ESPLINE®, INFLUENZA A&B-N (FUJIREBIO Inc., Tokyo, Japan) which is a highly sensitive kit for avian influenza virus [3]. All positive agents were identified by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests with specific antisera against influenza A virus strains as described elsewhere [15, 17]. Samples that were negative for the influenza virus by the detection kit were further tested by HI using specific antisera against in fluenza A virus strains as described elsewhere [15]. Samples that were negative for AIV and APMV isolation in each fiscal year were isolated (Table 1). From 2001 to 2008, the overall isolation rate of AIV was 0.95% and that for APMV was 0.30%. This rate for AIVs was similar to that in our previous surveillance in the same district since 1979 (1.9%, 129 isolates/6,801 fecal samples). Subtypes of the 41 strains of AIV isolated were characterized by HI and NI tests and were classified into 12 different subtypes: H1N1, H3N9, H4N6, H5N3, H6N1, H6N2, H6N5, H6N8, H9N2, H9N6, H10N4 and H11N9 (Table 2). Predominant combinations of HA and NA subtypes were H4N6 (14 iso-

<table>
<thead>
<tr>
<th>Species</th>
<th>Total AIV and APMV isolation in each fiscal year</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whistling swan</td>
<td>10/3/291</td>
<td>28/4/1890</td>
</tr>
<tr>
<td>Duck spp.</td>
<td>5/0/212</td>
<td>8/4/1476</td>
</tr>
<tr>
<td>Mallard</td>
<td>5/0/12</td>
<td>2/1/471</td>
</tr>
<tr>
<td>White-fronted goose</td>
<td>0/0/11</td>
<td>0/2/189</td>
</tr>
<tr>
<td>Common teal</td>
<td>0/0/6</td>
<td>3/0/139</td>
</tr>
<tr>
<td>Eurasian wigeon</td>
<td>0/0/11</td>
<td>0/1/106</td>
</tr>
<tr>
<td>Northern pintail</td>
<td>0/0/31</td>
<td>0/0/36</td>
</tr>
<tr>
<td>Black-tailed gull</td>
<td>0/0/26</td>
<td>0/0/26</td>
</tr>
<tr>
<td>Gadwall</td>
<td>0/1/2</td>
<td>0/1/2</td>
</tr>
</tbody>
</table>

| Total                    | 18/3/658                                      | 41/13/335 |
| Isolation rate (%)       | 2.74/0.46                                     | 0.95/0.30 |

a) Number of AIV isolates/APMV isolates/total tested samples.
b) Isolation rate of AIVs/APMVs.
lates) and H6N8 (7 isolates). Isolation of H3N9 and H9N6 subtypes, which were isolated in 2001 and 2008, respectively, has not been reported in the field in Japan.

A large number of H5N1 HPAI viruses have been isolated from swan species in European countries and Mongolia since 2005, and HPAI outbreaks in poultry caused by H5N1 viruses have been reported after isolation from swans [2]. These incidents indicate that swans play a key role in the spread of HPAI viruses in Europe. In addition, in April and May 2008, H5N1 HPAI viruses were isolated from dead or moribund whooper swans (Cygnus cygnus) in Aomori, Akita and Hokkaido Prefectures in northern Japan [18, 19]. The present study showed that whistling swans had the largest number of AIVs among all tested species (Table 2). Our findings suggest that HPAI viruses may have been introduced into the San-in district via the migration of wild swans.

Western Japanese poultry farms experienced two outbreaks of HPAI viruses caused by H5N1 subtypes in 2004 and 2007. Before the outbreaks, we isolated two H5 subtypes of AIV, designated A/teal/Tottori/150/2002 (H5N3) and A/whistling swan/Shimane/580/2002 (H5N3) (GeneBank Accession numbers of HA gene: AB535130 and AB535131, respectively) in the field specimens. Sequencing analysis of HA genes revealed that the deduced amino acid sequence at the cleavage site of both isolates showed typical avirulent motifs (RETR/G), indicating that they were presumably low or non-pathogenic AIVs. Phylogenetic analysis based on nucleotide sequences of the HA gene segment showed that A/teal/Tottori/150/2002 and A/whistling swan/Shimane/580/2002 belonged to Eurasian lineages and clustered in a different branch with the H5N1 HPAIVs that were isolated in Japan in 2004, 2007 and 2008 (Fig. 1). Both isolates were also separate from the H5N2 subtype of Japanese LPAIs isolated from chicken farms in 2005. Ito et al. [7] demonstrated that a non-pathogenic AIV strain, A/whistling swan/Shimane/499/83 (H5N3), developed into a highly pathogenic virus showing 100% mortality in chickens after several rounds of infection in chickens. This suggests that circulation of non-pathogenic field isolates in chickens can lead to the development of HPAI variants. In fact, H5N2 LPAIVs isolated in the Unites States in 1983 or Mexico in 1993 to 1995 increased in pathogenicity and emerged in poultry as HPAIVs [5, 8]; however, which H5 low pathogenic isolates from wild birds adapt and evolve to become highly pathogenic to chickens is still unclear. Therefore, in order to understand this issue, experimental investigations using both H5 isolates in the present study is considered to be carried out.

In the present study, 13 APMVs were also isolated from wild birds (Table 1). To determine the APMV serotypes, a set of antisera against APMV-1 to -9 is essential. Because we did not have access to antisera for all serotypes, the isolated APMVs could not be serotyped. The HI test using anti-APMV-1, -2 and -4, as described above, confirmed that 5 of 13 isolates were positive only for APMV-1 antisera, indicating that these isolates may be NDV strains. On the other hand, of the remaining 8 isolates, four isolates were positive only for APMV-2 and the remainders were positive only for APMV-4 antisera. In Japan, from 2001 to 2007, NDVs, including virulent pathotypes for chicken, were sporadically found in wild birds and poultry [9]. Among these, several Japanese isolates were genetically similar to Korean isolates, suggesting that they were derived from an immediate common ancestor. As a possible explanation for viral introduction into Japan, it was considered that the movement of wild birds may be related to viral dissemination [9]; therefore, to better understand the epidemiology of NDVs in wild birds, further genetic and serological studies of the APMV isolates in this study are necessary.

Our data indicate that various subtypes of AIV and APMV are prevalent in numerous species of migratory wild birds flying into the San-in district of western Japan. Continued surveillance over multiple years will allow for a better understanding of the role of wild birds in the introduction and dissemination of AIVs and APMVs in the field. Therefore, it is necessary to continue the surveillance of these serious poultry pathogens in migratory waterfowl and shorebirds.

Table 2. Antigenic characterization of AIV isolates from fecal samples from waterfowl and shorebirds in the San-in district of Western Japan from 2001 to 2008

<table>
<thead>
<tr>
<th>Species</th>
<th>Total no.</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whistling swan</td>
<td>28</td>
<td>H3N9 (2)*</td>
<td>H1N1 (2)</td>
<td>H1N1 (1)</td>
<td>NI</td>
<td>H4N6 (6)</td>
<td>NI</td>
<td>NI</td>
<td>H6N1 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H6N8 (6)</td>
<td>H5N3 (1)</td>
<td>H6N8 (1)</td>
<td>H6N8 (1)</td>
<td>H6N8 (1)</td>
<td>H1N1 (1)</td>
<td>H1N1 (1)</td>
<td>H9N2 (1)</td>
</tr>
<tr>
<td>Mallard</td>
<td>2</td>
<td>NI*</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>H9N6 (1)</td>
</tr>
<tr>
<td>Common teal</td>
<td>3</td>
<td>H4N6 (1)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>H11N9 (1)</td>
</tr>
<tr>
<td>Duck spp.</td>
<td>8</td>
<td>NI</td>
<td>H4N6 (1)</td>
<td>H4N6 (1)</td>
<td>H4N6 (1)</td>
<td>H6N2 (1)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

a) Number of isolates of each antigenic subtype is shown in parentheses.
b) No viruses were isolated.
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Fig. 1. Phylogenetic tree for HA gene in recent H5 isolates. The phylogenetic tree was generated using the neighbor-joining method and bootstrap testing (n=1,000) in MEGA (4.0.2). Analysis was based on nucleotides 105–1659 (1555 bp) of the HA gene. ● represents isolates in Japan in 2004, ■ in 2005, ○ in 2007 and ▲ in 2008. H5 isolates in this study are underlined.
REFERENCES


